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Report

Sex- and Strain-Specific Expression and Vomeronasal Activity of Mouse ESP Family Peptides

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Summary

Male mice secrete exocrine-gland-secreting peptide 1 (ESP1) from the extraorbital lacrimal gland into tear fluid [1]. Other mice detect ESP1 through sensory neurons in the vomeronasal organ (VNO), a secondary olfactory system that senses pheromonal information, including sex, strain, and species. ESP1 is now known to be a member of a multigene family that encodes peptides of various lengths. We herein performed genomic and expression analyses of the ESP family. The ESP family consists of 38 members in mice and 10 members in rat but is absent from the human genome, suggesting rapid molecular evolution. In addition to the male-specific ESP1, we discovered one, which we designated ESP36, that, in adult BALB/c mice, is expressed only in the female extraorbital lacrimal gland. The sexually dimorphic expression is ensured by the release of testosterone after puberty. However, we observed dramatic differences in the expression levels of ESPs between strains. Finally, all ESPs elicited an electrical response in the vomeronasal epithelium but not in the main olfactory epithelium. Multielectrode recording of VNO activity demonstrated that ESP1 induces action potentials in vomeronasal neurons, leading to an increase in the spike firing rate, and that ESP1 is recognized by narrowly tuned vomeronasal sensory neurons. Sexual dimorphism and strain differences of ESPs and their reception in the VNO suggest that the ESP family can convey information about sex and individual identity via the vomeronasal system. The chemosensation of this nonvolatile peptide family by direct contact appears to be one of strategies for sociosexual communication in rodent species.

Results and Discussion

Many animal species utilize external chemical cues called pheromones to send social and sexual information. In mice, pheromones are detected by two olfactory sensory circuits, the main olfactory pathway and the vomeronasal system, leading to specific behavioral and endocrinological outputs [2, 3]. Surgical ablation of the vomeronasal organ (VNO) results in abnormal sociosexual behavior [4, 5]. In addition, mutant mice lacking components involved in the vomeronasal pathway showed unusual social and reproductive behaviors [6–8]. Thus, the VNO appears to play an important role in sexual behavior and social aggression in mice.

Recently, we identified a male-specific 7 kDa peptide produced in the extraorbital lacrimal gland (ELG) and released in tear fluid [1]. This peptide, which we named exocrine-gland-secreting peptide 1 (ESP1), stimulates the expression of c-Fos in a small number of basal vomeronasal sensory neurons (VSNs) in female mice [1]. Thus, although the pheromonal action of ESP1 in male-female communication remains to be elucidated, ESP1 is a candidate for a vomeronasal receptor type 2 (V2R) ligand that acts as a pheromone in mice. In addition, ESP1 turns out to be a member of a previously unrecognized multigene family reported to consist of 24 homologous genes [1], suggesting that the ESP family can provide a basis for variation in the patterns of information transmitted between individuals, sex, strains, or species.

In National Center for Biotechnology Information (NCBI) Mouse Build 36 (C57BL/6 strain), we have now identified a total of 38 ESP family genes, one of which has not yet been mapped to a chromosome. The mouse ESP family is clustered on chromosome 17 within a 3.2 Mb region of the telomeric end of the major histocompatibility complex (MHC) class I loci (Figure 1A). We numbered the ESP family from ESP1, which is located at the most telomeric side of the cluster, increasing the number in the direction of the centromere. Interestingly, there is a very large cluster of olfactory receptor (OR) genes between the MHC class I and ESP regions (Figure 1A). There is some overlap between the MHC class I and OR clusters, whereas the OR and ESP clusters are clearly segregated. On the basis of the size of the unsequenced gap remaining in the ESP cluster region (Figure 1A), we expect that our current inventory of the ESP family is nearly complete.

A homology search on the currently available database revealed 10 ESPs in rat and one possible ESP in opossum but no ESP genes in the human genome, suggesting that the family could have originated in the common ancestor of mammals and that the ESP family has undergone extremely rapid molecular evolution. To examine the positional relationship of the ESP family, we constructed genomic maps based on the location of orthologous genes among mouse, rat, and human in the vicinity of the MHC region (*Gabbr1*, *Ubd*, and *Ors*) and of the ESP region (*Crisp1*, *Crisp2*, *Rhag*, and *Mut*) (Figure 1A). Unlike the mouse ESP family, the rat ESPs are not clustered next to the MHC region but rather on a different chromosome, suggesting that the boundaries of the ESP and MHC/OR gene clusters have been

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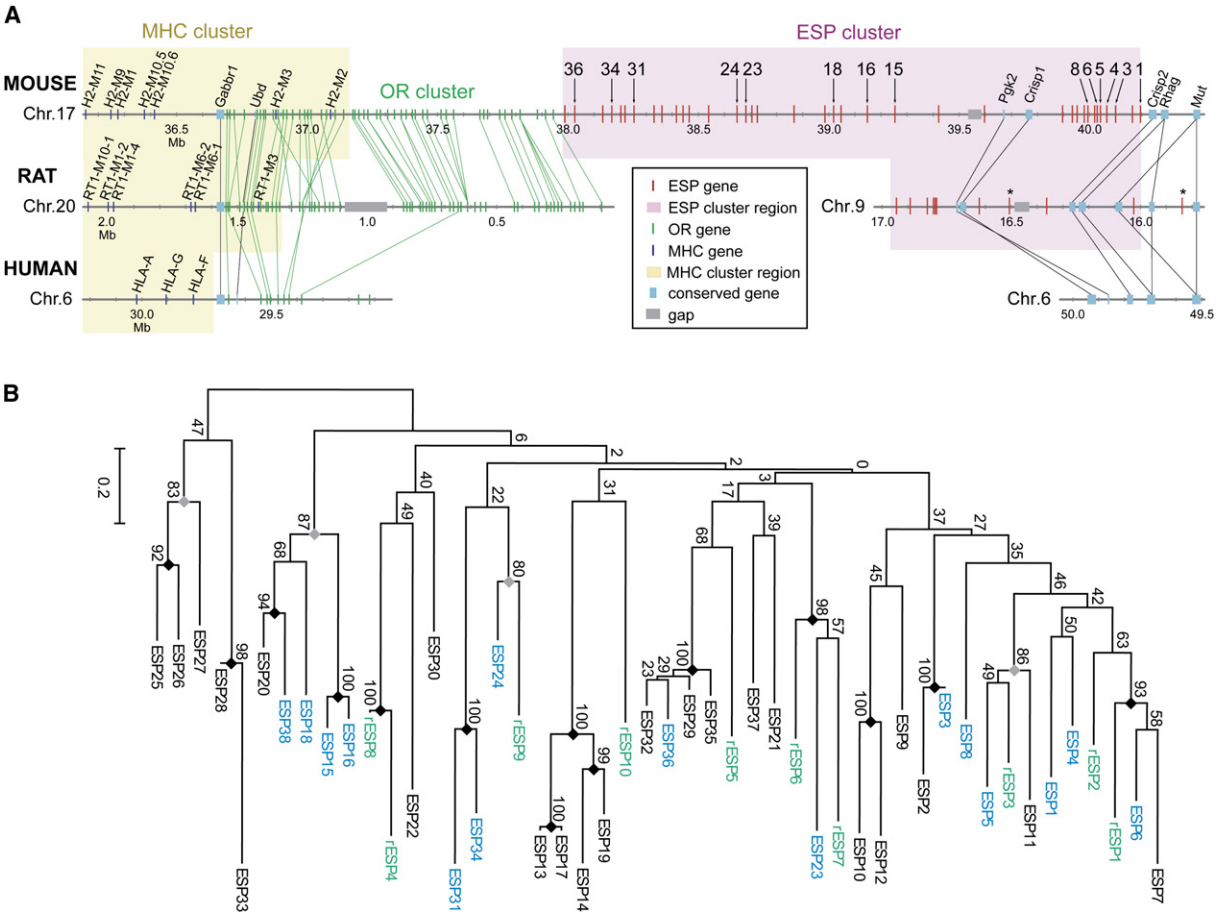

Figure 1. The ESP Gene Family

(A) Genomic location of the ESP gene family in various species. The locations of the last exons of ESP genes are shown as red lines. They are numbered starting from ESP1 up to ESP37, counting toward the centromere. The one that has not been mapped to a chromosome was numbered ESP38. The numbers of the expressing ESP genes are indicated. The genes conserved among mouse, rat, and human are shown as blue lines. Colored boxes represent the ESP region (pink) and the MHC cluster (yellow). OR genes are shown in green. Gaps are shown in gray boxes. The two rat ESP genes indicated with an asterisk are identical, possibly because of an error in the database.

(B) Phylogeny of ESP genes in mouse and rat. The tree was reconstructed with the neighbor-joining method with Poisson-corrected protein distances. Squares indicate nodes whose bootstrap values are at least 90% (black) and 70% (gray). The expressing ESP genes in mouse are indicated in blue. The rat ESP genes are shown in green. The scale bar represents 0.2 amino acid substitutions per site.

subjected to genomic rearrangements after the mouse-rat speciation.

Of the 38 mouse ESPs, 14 appear to be pseudogenes: Seven ESPs (ESP12, 14, 19, 29, 32, 35, and 37) lack a signal sequence and the first Met codon, and seven have a signal sequence but possess a stop codon in the signal sequence (ESP10, 11, 13, 17, and 33) and/or within the first ten amino acids after the potential signal-sequence cleavage site (ESP7, 11, 21, and 33). Thus, from the genomic sequence, 24 out of 38 ESPs would be predicted to encode intact ESP peptides. The peptides range from 60 to 160 amino acids in length, and, after a signal sequence, they contain an N-terminal domain of 10–15 amino acids with higher homology than other regions (Figure S1 in the Supplemental Data available online).

To explore the evolutionary relationships of the mouse and rat ESPs, we carried out phylogenetic analysis by using the neighbor-joining method [9]. The mouse and rat ESPs are interspersed throughout the tree, suggesting that the diversification occurred before the mouse-rat separation (Figure 1B). There were some mouse-specific clades in the phylogenetic tree of ESPs: (1) mouse ESP13, 14, 17, and 19 and (2) mouse ESP29, 32, 35, and 36. This suggests that a small expansion might have occurred in mouse lineage. There was some orthologous relationship between mouse and rat: rat ESP (rESP) 1 and ESP6 (49% identity), rESP3 and ESP5 (61% identity), rESP7 and ESP23 (48% identity), and rESP9 and ESP24 (68% identity). The ESPs in the two mouse-specific clades are in a part of the genome that is lacking in the rat.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis with BALB/c mice revealed that 15 out of 24 intact ESP genes were expressed in the ELG, Harderian gland (HG), and/or submaxillary gland (SMG) of sexually mature mice (Figure 2A). We determined the full-length complementary DNA (cDNA) sequences of the 15 expressed ESPs by 5′- and 3′-RACE (rapid amplification of cDNA ends) (Figure S2). The ESPs consist of three exons, except for ESP5 and ESP36, which possess five and four exons, respectively.

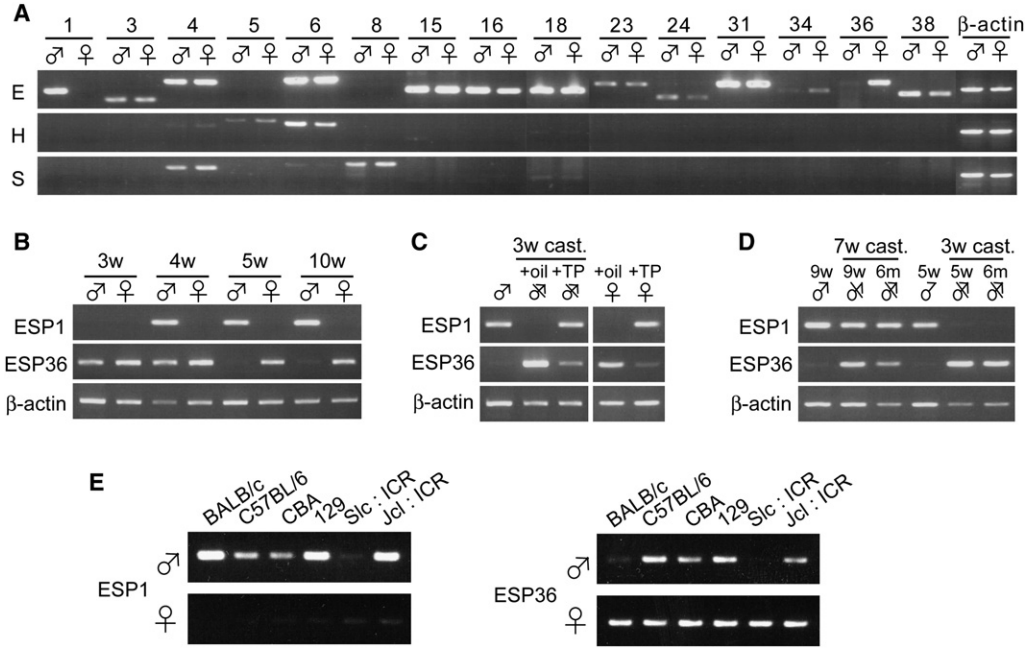

Figure 2. Expression Profiles of the ESP Gene Family

(A) Expression profile of the ESP genes in BALB/c mice as determined by RT-PCR in three exocrine glands. The expression of ESP1 and ESP36 represents sex-specific patterns. The expression of ESP genes in the extraorbital lacrimal gland (E), Harderian gland (H), and submaxillary gland (S) in 10-week-old male or female BALB/c mice was analyzed by RT-PCR.

(B) Sexually dimorphic expression profiles of ESP1 and ESP36 at different ages in BALB/c mice. Before 4 weeks of age (puberty), both male and female mice express ESP36 but not ESP1. Around 4–5 weeks of age, the expression of ESP1 and ESP36 in male mice appears to be up- and downregulated, respectively. This results in the sexually dimorphic expression patterns in mature mice.

(C) Administration of testosterone propionate (TP) to castrated (3w cast.) mice (every day for 2 weeks) induced ESP1 expression and significant downregulation of ESP36 expression. Administration of TP to female mice (every day for 3 weeks from 3 to 5 weeks of age) elicited the expression of these two genes in a pattern similar to that of male mice. At 5 weeks of age, the expression of ESP1 or ESP36 was examined.

(D) Investigation of androgen dependency of the sex-specific expression of ESPs in BALB/c mice. Male mice (7 or 3 weeks old) were castrated, and at 5 weeks (5w), 9 weeks (9w) or 6 months (6m), the expression of ESP1 or ESP36 was examined. The expression of ESP1 was not affected by castration at 7 weeks, but male mice castrated at an age of 3 weeks showed expression patterns similar to those of female mice.

(E) Strain differences in expression profiles of ESP1 and ESP36 in various mouse strains. The expression of ESP1 and 36 genes in the extraorbital lacrimal gland in 10-week-old male or female BALB/c, C57BL/6, CBA, 129, Slc:ICR, and Jcl:ICR mice was analyzed by RT-PCR.

The start codon and a putative signal peptide sequence are located in the second from the last exon, and the mature peptide following the signal-sequence cleavage site is encoded on the last exon: In most cases, the first Met is found in exon 2, and the processed peptide is coded on exon 3. Although the actual secreted mature ESPs might be shorter than the predicted peptides after processing, as in the case of ESP1 [1], the size of the predicted ESPs varies from 5 to 15 kDa.

Within the 38 ESPs found in the mouse genome, we discovered one, which we designated ESP36, that, in adult BALB/c mice, is expressed only in females (Figure 2A). Before puberty, both male and female mice express ESP36 but not ESP1 (Figure 2B). The expression of ESP1 and ESP36 in the male mice appears to be up- and downregulated, respectively, around 4–5 weeks of age, resulting in the sexually dimorphic expression pattern in mature mice (i.e., ESP1 in male and ESP36 in female) (Figure 2B). These results led to a hypothesis that the expression of ESP1 and ESP36 is modulated by androgen released during puberty in BALB/c mice.

To investigate androgen dependence of the sex-specific ESPs, we castrated 3-week-old male BALB/c mice and examined the expression of ESP1 and ESP36 2 weeks later. Sham-operated mice showed a sexually dimorphic expression pattern, whereas the castrated male mice showed an expression pattern similar to that of female mice, such that no ESP1 was expressed but ESP36 was still expressed (Figure 2C). When castrated mice were administered testosterone propionate every day for 2 weeks, ESP1 expression was induced, and ESP36 expression was significantly downregulated. Female mice treated with testosterone every day from 3 to 5 weeks of age exhibited an expression pattern similar to that of male mice. These results clearly demonstrate the testosterone-dependent expression of ESP1 and ESP36.

Interestingly, the effect of castration on ESP1 expression was not observed in mature adult male mice. Thus, when 7-week-old male mice were castrated, ESP36 expression was induced, whereas ESP1 expression was not downregulated at 9 weeks or 6 months of age (Figure 2D). These results suggest that the increase in testosterone leads to the constitutive expression of ESP1, and therefore, even with castration, ESP1 continues to be expressed. This possibility was further supported by the fact that just a few weeks of testosterone treatment was enough to ensure continuous ESP1 expression in both male and female mice (data not shown).

These observations are of particular interest in terms of the hormonal modulation of epigenetic dynamics at the level of genomic structure.

To determine whether there are differences in sequences between mouse strains, we compared 15 expressed ESP genes between BALB/c and C57BL/6 mice. Of the 15 ESPs, 13 had exactly the same sequences, whereas ESP34 and 36 had some strain differences: ESP34 had 96.5% identity between the two strains at the DNA-sequence level, and ESP36 in C57BL/6 mice was 24 amino acids longer than that in BALB/c mice because of a single base deletion in BALB/c (Figures S1 and S2). In addition, there were some differences in the expression levels as determined by RT-PCR (Figure 2E). ESP1 was expressed in BALB/c and 129 mice, whereas ESP1 expression in C57BL/6 and CBA strains was much lower. Slc:ICR strains showed no expression of ESP1, whereas we found robust expression in Jcl:ICR mice. ESP36 was female specific in BALB/c and Slc:ICR adult mice but not in C57BL/6, CBA, 129, or Jcl:ICR mice (Figure 2E). The results suggest that there is some degree of difference between strains in both noncoding and coding regions of the ESP family, resulting in not only differences in the peptide sequences but also differences in the relative ratio of expression between mouse strains.

We next examined the hypothesis that the ESP family comprises a repertoire of VNO ligands. We have previously demonstrated that the electrical response of the VNO to recombinant ESP1 saturated at a concentration of 10^{-7} M [1]. We produced recombinant peptides for the additional 14 expressed ESPs in *E. coli*, purified them to homogeneity, and tested their activities by electrovomeronasogram (EVG) (Figure 3A). All the recombinant ESPs evoked a negative field potential in the female VNO at 10^{-7} M, suggesting that the ESP signal was converted to an electronic event in the VNO (Figure 3B). In contrast, other proteins such as odorant-binding proteins did not elicit a response in the VNO. Furthermore, none of the ESPs evoked a negative field potential in the main olfactory epithelium (MOE) in the electro-olfactogram (EOG) recording experiments (Figure 3C). As a positive control, 2-heptanone and MHC peptides showed responses in both the VNO and MOE, in agreement with the previous results (Figures 3B and 3C) [10–12]. In addition, we found that a recombinant major urinary protein (MUP1) also elicited an electrical response in the VNO (Figure 3B). These results suggest that the ESP family members are detected as ligands for the VNO system in VSNs.

Next, we examined the responses to ESPs at three different positions (i.e., anterior, posterior, and between) in the VNO of the same mouse by applying three different ESPs sequentially from a three-barrel glass capillary (Figure S3). Every time we moved the recording electrode to a new site, we obtained different amplitudes and specificities for different ESPs, suggesting that the population of neurons expressing a receptor for each ESP is different from position to position and from mouse to mouse. These observations are reasonable assuming that single vomeronasal neurons express single vomeronasal receptors [13, 14] and that, based on the distribution of neurons expressing a given single vomeronasal receptor, one recording area of local field

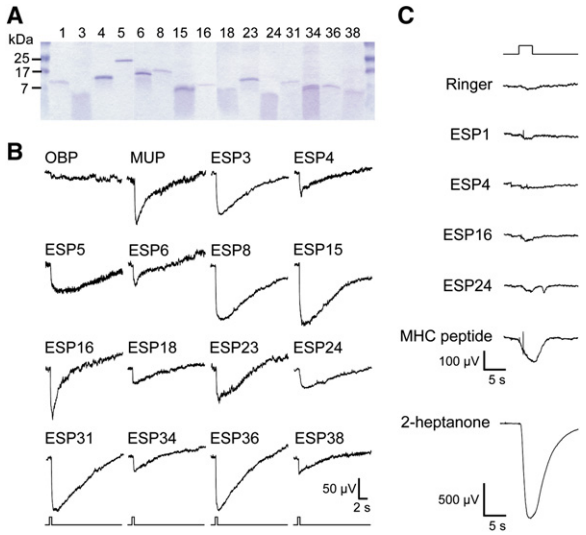

Figure 3. EVG and EOG Responses by Recombinant ESP Peptides

(A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the recombinant ESP peptides. The numbers above the gel indicate the ESP numbers.

(B) EVG responses to the recombinant ESP peptides in the VNO. The concentrations of proteins or peptides were 10^{-7} M. Results are representative of three to four recordings for each ESP from 42 mice.

(C) EOG responses induced by MHC peptide and 2-heptanone but not to the recombinant ESP peptides in the MOE. The concentrations of proteins or peptides were 10^{-7} M. Results are representative of two to five recordings for each peptide from six mice. It is of note that the sample delivery method in EOG and EVG recording is different (see the Supplemental Experimental Procedures).

potential could be expected to contain at most one or few neurons that express the target ESP receptor.

So that the responsiveness of VSNs to ESPs at the single-neuron level could be further explored, the spiking activity of VSNs was recorded with a multielectrode array with 60 extracellular electrodes [15]. Stimuli, including ESP1, ESP4, ESP6, ESP8, ESP31, and MUP1 (each at 10^{-7} M) as well as urine from male and female BALB/c mice (1:100), were diluted in Ringer's solution. In the nine preparations, a small percentage of electrodes recorded from ESP1-responsive neurons (Figure 4A). Out of a total of 320 identified single units, five (1.6%; one neuron detected in each of five different preparations, including three from female mice and two from male mice) clearly responded to ESP1 (Figure 4C). By the same quantitative criteria (see the Supplemental Experimental Procedures), no cells responded to the negative control (Ringer's). This probability (1.6%) is fairly consistent with the relative population of V2Rp, a receptor for ESP1, in the vomeronasal epithelium [1]. Notably, none of the five ESP1-detecting cells responded to dilute urine from either sex or other ESPs. Of the 320 neurons, 20 and 27 responded to female and male mouse urine, respectively; none of these responded to any of the tested ESP peptides (ESP4 and ESP6: 320 single units; ESP8 and ESP31: 122 single units) nor to recombinant MUP1 (320 units), at 10^{-7} M concentration (Figure 4D). One likely interpretation for these results is that these other ESPs are detected by more-rarely expressed receptor types that were not among the neurons sampled by the multielectrode array. Nonetheless, these results

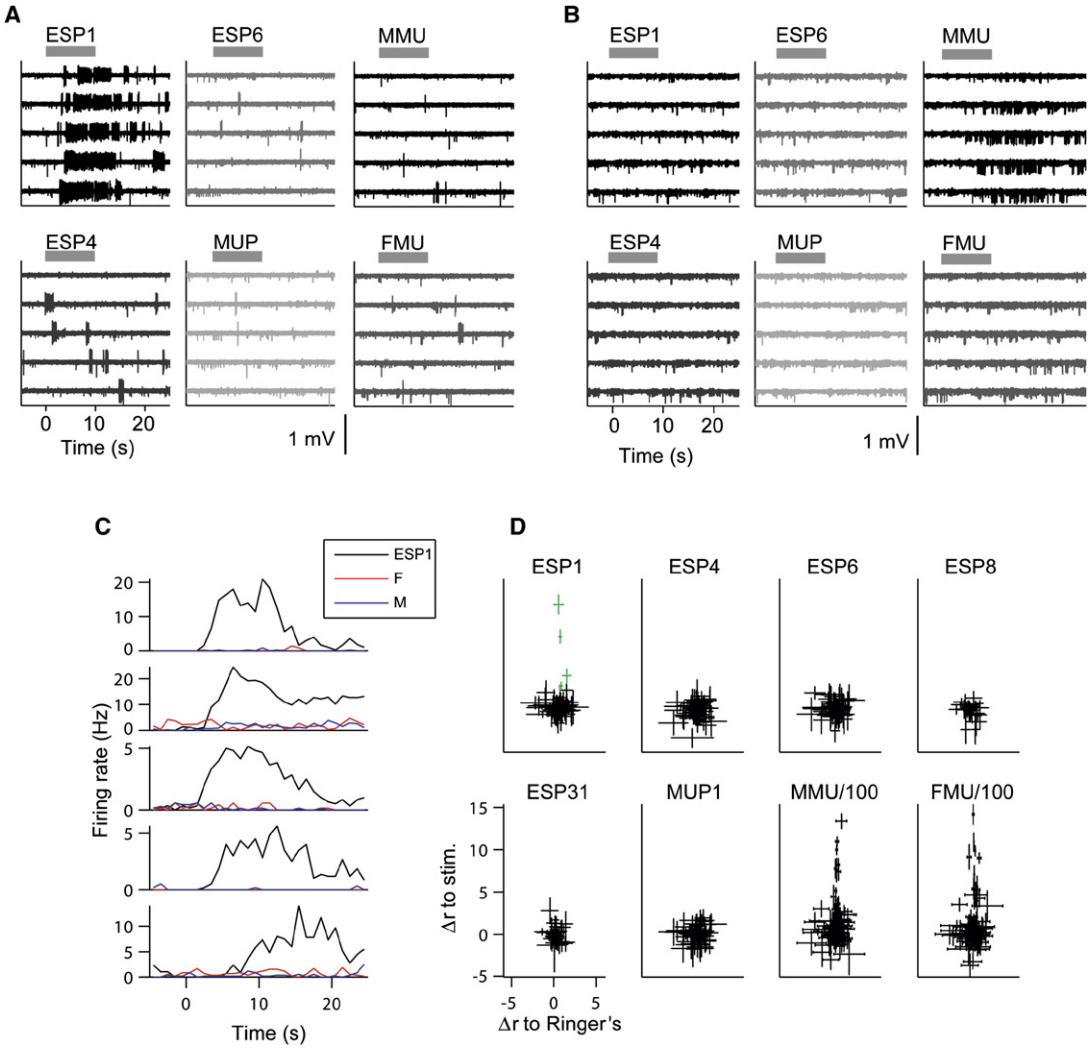

Figure 4. VSNs Fire Action Potentials when Stimulated with ESP1

Extracellular voltage recorded on one electrode in response to five interleaved presentations each of ESP1, ESP4, ESP6, MUP1 (all at 10^{-7} M), and 100-fold dilute male (MMU) and female (FMU) mouse urine. The stimulus delivery period is indicated by a gray bar (top left).

(A) A single unit responding to ESP1 but not to other stimuli. Within a panel, each voltage trace corresponds to a separate stimulus trial.

(B) A single unit responding to MMU but not to other stimuli.

(C) Average firing rate as a function of time (using 1 s bins) for five ESP1-responsive neurons (recorded in five different preparations). Different stimuli are indicated with different colors as shown in the legend. "F" indicates BALB/c female mouse urine (1:100), and "M" indicates BALB/c male mouse urine.

(D) Average firing rate change, Δr, in response to stimulation by ESPs, MUP1, or dilute urine stimulus (along the vertical axis) compared against the cell's response to Ringer's control (horizontal axis). Each panel represents 320 neurons recorded in nine preparations, except for ESP8 and ESP31 with 122 neurons in three preparations. Error bars indicate the standard error of the mean (SEM) across trials. In the ESP1 panel, neurons shown in (C) are highlighted in green.

demonstrate that ESP1 indeed elicits a neural spike at the axon level in a small subset of VSNs, and the receptor expressed by each neuron appears to be narrowly tuned to its cognate ligand.

In this study, we have addressed the following specific questions: (1) How is the ESP family conserved across various mammalian species? (2) Is there sexual dimorphism in the expression of the ESP family members? (3) What about differences between strains? And (4) do all the ESP members stimulate the VNO? To answer these questions, we performed genomic and expression analyses of the ESP family, and we found the ESP family in rodent species but not in human, and testosterone-induced sexual dimorphism of ESPs. There were some differences in the relative ratio of expression of ESPs between mouse strains. Examining expression patterns in wild mice should tell us whether these differences represent differences between individuals. Finally, the functional characterization of recombinant ESPs by electrophysiological recording suggested that the ESPs comprise a VNO ligand repertoire. The results implicate rapid molecular evolution of the ESP family and its function via the vomeronasal system, providing insight into peptide-mediated sociosexual communication. Further studies will reveal how the ESP signal is received by the

accessory olfactory bulb, which is the first relay center of the vomeronasal system, and how the higher neuronal network generates neuroendocrine or behavioral output.

Supplemental Data

Experimental Procedures, three figures, and one table are available at http://www.current-biology.com/cgi/content/full/17/21/1879/DC1/.

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Accession Numbers

The sequences reported in this paper have been deposited in the GenBank database under accession numbers AB306980–AB307019 (mouse), and AB307020–AB307029 (rat).